E. COLI O157:H7 C1 ESTERASE INHIBITOR-BINDING PROTEIN AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application No. 60/243,675, filed October 26, 2000.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with United States government support awarded by _____.

BACKGROUND OF THE INVENTION

[0003] Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 strain is a human enteric bacterial pathogen that causes diarrheal disease, hemorrhagic colitis, and hemolytic uremic syndrome (HUS). Each year in the United States, an estimated 20,000 people suffer from diarrheal disease associated with E. coli O157:H7 infection, which is typically contracted by ingesting contaminated foods, especially undercooked meat. Approximately 6% of infected individuals develop HUS, which can lead to renal failure and death. Young children and the elderly are particularly susceptible to developing HUS.

[0004] In general, bacterial infections are commonly treated by administering appropriate antibiotics. However, *E. coli* O157:H7 infection typically has a very rapid progression, and is consequently very difficult to treat. Often by the time the disease is diagnosed, the infected individual is severely ill and toxic proteins secreted by the bacteria may have damaged mucosal cells and entered the blood stream. Antibiotic treatment of patients infected with *E. coli* O157:H7 is generally not successful, and is now believed to be contraindicated.

[0005] *E. coli* O157:H7 bacteria are very proficient at establishing an infection; ingestion of as few as 10 live bacteria is sufficient to establish an infection. The highly

infective nature of *E. coli* O157:H7 and the devastating sequelae associated with infection by this bacteria, together with the extensive public attention given to outbreaks of hemorrhagic colitis, has generated a great deal of interest among medical professionals and the general public in developing the means for early diagnosis and treatment of the disease. The entire genome of the *E. coli* O157:H7 EDL933W (ATCC 43895) was sequenced with the expectation that valuable information concerning the organism's pathogenicity would be uncovered, which may facilitate development of methods of preventing infections, or preventing or treating hemolytic uremic syndrome in individuals infected with of the organism. The DNA sequence of *E. coli* O157:H7 was compared with that of *E. coli* K12, a non-pathogenic strain commonly used in research. The genome of *E. coli* O157:H7 exceeds that of *E. coli* K-12 by more than a million base pairs and has up to 1000 genes not found on K-12. These additional gene sequences are distributed throughout more than 250 sites in islands, with each island containing from zero to sixty genes (1).

[0006] What is needed in the art is an improved understanding of factors involved in the pathogenesis of *E. coli* O157:H7, and methods for preventing or treating hemolytic uremic syndrome in individuals infected with *E. coli* O157:H7. What is also needed in the art is an understanding of factors responsible for enteric disease that are common to pathogenic *E. coli*.

BRIEF SUMMARY OF THE INVENTION

[0007] The present invention provides an isolated polypeptide comprising the amino acid sequence of amino acid residues 230-630 of SEQ ID NO:2.

[0008] In another aspect, the present invention provides an isolated polypeptide comprising an amino acid sequence having at least 70% amino acid identity to amino acid residues 24-886 of SEQ ID NO:2, the polypeptide comprising a sequence corresponding with and identical to amino acids 434-444 of SEQ ID NO:2 and having the ability to bind to and cleave C1 esterase inhibitor.

[0009] In another embodiment, the present invention provides an isolated polypeptide, the amino acid sequence of which comprises at least 17 consecutive amino acid residues of SEQ ID NO:2.

[0010] In yet another aspect, the present invention provides a genetic construct comprising a polynucleotide encoding a polypeptide comprising the amino acid sequence of amino acid residues 24-886 of SEQ ID NO:2 or a sequence complementary thereto, the polynucleotide operably linked to an expression control sequence. Additionally, the present invention is drawn to a cell comprising the genetic construct.

[0011] In another embodiment, the present invention includes an antibody that binds specifically to an antigenic determinant found on a polypeptide comprising an amino acid sequence comprising SEQ ID NO:2, preferably to an epitope in the C1 esterase inhibitor binding domain of the polypeptide.

[0012] In another aspect, the present invention provides a method of preventing or treating colitis or hemolytic uremic syndrome in a subject infected with an enterohemorrhagic pathogen expressing an inhibitor protein comprising an amino acid sequence substantially homologous to SEQ ID NO:2, the inhibitor protein being capable of reducing the activity of C1 esterase inhibitor, comprising administering to the subject an antibody that binds specifically to an antigenic determinant found on a polypeptide having the amino acid sequence of SEQ ID NO:2, or a chelator capable of binding divalent cations, the antibody or chelator administered in an amount effective to reduce proteolytic inactivation of C1 esterase inhibitor by the inhibitor protein.

[0013] Another aspect of the invention provides a method of preventing colitis or hemolytic uremic syndrome in a subject at risk for infection with an enterohemorrhagic pathogen expressing an inhibitor protein comprising an amino acid sequence substantially homologous to SEQ ID NO:2, the inhibitor protein being capable of reducing the activity of C1 esterase inhibitor, comprising administering to the subject an inactivated polypeptide comprising at least one antigenic determinant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2 in an amount effective to induce an immune response.

[0014] In another aspect, the invention provides a method of preventing or treating colitis and/or hemolytic uremic syndrome in a subject infected with an enterohemorrhagic pathogen expressing an inhibitor protein comprising an amino acid sequence substantially homologous to SEQ ID NO:2, the protein inhibitor being capable of reducing the activity of C1 esterase inhibitor, comprising the step of administering to the subject C1 esterase inhibitor in an amount effective to reduce activation of at least one proteolytic cascade selected from the group consisting of classical complement cascade, intrinsic coagulation pathway, and kinin-forming system.

[0015] The present invention provides a method of testing a molecule for the ability to reduce proteolysis of C1 esterase inhibitor by an inhibitor protein comprising an amino acid sequence substantially homologous to amino acid residues 24 to 886 of SEQ ID NO:2, the inhibitor protein being capable of proteolyzing C1 esterase inhibitor, comprising combining the inhibitor protein, test molecule, and C1 esterase inhibitor under suitable conditions for a period of time sufficient to allow inhibitor protein-C1esterase inhibitor interaction; and comparing the level of the C1 esterase inhibitor or C1 esterase inhibitor activity to that of a control lacking the test molecule.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0016] Fig. 1 shows the differential effect of E. coli strains containing (Fig. 1A and Fig. 1C) or lacking (Fig. 1B and Fig. 1D) the plasmid pO157 on aggregation of T cells.

[0017] Fig. 2 shows stained proteins separated by SDS/PAGE.

[0018] Fig. 3 shows that synthesis of StcE correlates with the presence of pO157.

[0019] Fig. 4 shows that C1 inhibitor in human serum is cleaved by StcE.

[0020] Fig. 5 shows cleavage of C1 inhibitor over time.

[0021] Fig. 6 shows differential cleavage of C1-INH by StcE and elastase.

[0022] Fig. 7 is an immunoblot showing a StcE-reactive band in fecal filtrates.

[0023] Fig. 8A shows that StcE E435D-His is unable to cleave C1-INH; Fig. 8B shows that StcE E435D-His is unable to bind C1-INH.

[0024] Fig. 9 shows amplification of three *stc*E-specific sequences from all *E. coli* isolates containing the pO157, and amplification of two of the three *stc*E-specific sequences.

[0025] Fig. 10 shows an immunoblot of culture supernatants probed with a polyclonal antibody to StcE.

[0026] Fig. 11 shows an immunoblot of C1-INH following incubation with culture supernatants, probed with anti-C1-INH antibody.

DETAILED DESCRIPTION OF THE INVENTION

[0027] Strains of the serotype O157:H7 EDL933W carry a 92 kb plasmid designated pO157. As described in the Examples below, bacterial strains containing the plasmid cause the aggregation of two cultured human $CD4^+$ T cell lines, Jurkat and MOLT-4, but not a B cell lymphoma line (Raji), or macrophage-like cell lines (U937 and HL-60). Aggregation of the cells occurs in the presence of serum, but not in the absence of serum. Strains lacking the plasmid do not cause aggregation. We employed transposon mutagenesis to identify a gene on pO157 of previously unknown function whose product is associated with the observed aggregation effect. The coding sequence and the deduced amino acid sequence of the protein it encodes are shown in SEQ ID NO:1 and SEQ ID NO:2, respectively. The protein, designated "StcE", contains a putative cleavable N-terminal signal sequence, is secreted into the culture medium, and is a protease that cleaves C1 esterase inhibitor, a protease inhibitor that controls the activation of C1, the first component of the complement cascade.

[0028] As one of skill in the art would appreciate, a protein comprising an amino acid sequence having minor substitutions, deletions, or additions from that of SEQ ID NO:2 would be suitable in the practice of the present invention. Conservative amino acid substitutions are unlikely to perturb the protein's secondary structure and interfere with its activity. SEQ ID NO:2 includes the N-terminal signal sequence, which although

expressed, is unlikely to be found on an isolated polypeptide. The expressed StcE protein likely undergoes post translational modification that results in cleavage of the N-terminal signal peptide. It is likely that StcE isolated from culture medium in which *stc*E positive strains are grown comprises amino acid residues 24-886 of SEQ ID NO:2. As shown in the Examples below, it appears that the 5' region of the StcE gene and promoter is less highly conserved than the 3' region of the gene.

[0029] It is specifically envisioned that isolated polypeptides having less than the full length sequence of amino acid residues 24-886 of SEQ ID NOL:2 will be useful in the practice of the present invention. StcE polypeptides that are truncated at the N-terminal or C-terminal regions and but have an intact metalloprotease binding domain corresponding to amino acid residues 434-444 of SEQ ID NO:2 may retain the ability to bind to and/or cleave the C1 esterase inhibitor. Binding and proteolytic activity of polypeptides can be evaluated using the methods set forth herein in the Examples, or by any suitable method. It is expected that StcE polypeptides that are truncated at the N-terminal or C-terminal regions or a polypeptide comprising an amino acid sequence comprising at least 17 consecutive amino acid residues of SEQ ID NO:2 may be used as an antigen against which antibodies specific for StcE may be raised. Preferably, the polypeptide comprises at least 25 consecutive amino acid residues of SEQ ID NO:2. More preferably still, the polypeptide comprises at least 40 consecutive amino acid residues of SEQ ID NO:2. One of ordinary skill in the art could easily obtain any of the various polypeptides comprising a portion of SEQ ID NO:2 by subcloning a sequence encoding the polypeptide into an expression vector, introducing the expression vector into a suitable host cell, culturing the cell, and isolating the expressed polypeptide using standard molecular biological techniques.

[0030] As used herein, an "inhibitor protein" is a polypeptide having substantial sequence identity with amino acid residues 24 to 886 of SEQ ID NO:2. As used herein, an inhibitor protein with substantial sequence identity to amino acid residues 24 to 886 of SEQ ID NO:2 comprises an amino acid sequence that has at least 70% amino acid identity to amino acid residues 24 to 886 of SEQ ID NO:2 and which has inhibitor protein activity. As used herein, "inhibitor protein activity" includes the ability to bind

to C1 esterase inhibitor, proteolyze C1 esterase inhibitor, and promote T cell aggregation in the presence of C1 esterase inhibitor. More preferably, an inhibitor protein of the present invention has 90% amino acid identity with amino acid residues 24-886 of SEQ ID NO:2; most preferably still, an inhibitor protein of the present invention has 95% amino acid identity with amino acid residues 24-886 of SEQ ID NO:2.

[0031] By an "isolated polypeptide" is meant a polypeptide that has been at least partially purified from contaminants and which is found other than in its natural state. For example, a isolated polypeptide could be obtained from a fecal sample by filtering the sample. One may obtain an isolated polypeptide using any partial purification of the polypeptide from the supernatant of a culture of bacterium that secrete the polypeptide, or from a bacterial cell lysate of bacteria that express but the polypeptide but do not secrete it.

[0032] In the examples below, polyclonal antibodies were raised against StcE protein in a band excised from a polyacrylamide gel. These antibodies were found to bind specifically to StcE protein. An antibody that binds specifically to an antigenic determinant is one that binds to a protein corresponding to a polypeptide comprising the amino acid sequence of amino acid residues 24-886 of SEQ ID NO:2, or to peptide fragments thereof, but does not bind significantly to other proteins. One of skill in the art will appreciate that using standard methods, one could raise monoclonal antibodies to a polypeptide comprising the amino acid sequence of amino acid residues 24-886 of SEQ ID NO:2 or peptide fragments, and that the monoclonal antibodies would also be useful in the practice of the present invention.

[0033] It is envisioned that an antibody preparation comprising at least one antibody directed against any of the antigenic determinants of the polypeptide of amino acid residue 24-886 could be used to passively immunize a person against a bacterium expressing a StcE protein. Preferably, the antibody preparation comprises an antibody that binds in the region of amino acid residues 434-444 of SEQ ID NO:2.

[0034] Because the StcE protein is a metalloprotease that requires zinc for its activity, it is envisioned that infections with bacteria expressing StcE or a related protein may be treated by delivering an agent capable of binding to a divalent cation in an amount effective in preventing or treating colitis or hemolytic uremic syndrome in a patient infected with an enterohemorrhagic pathogen expressing an inhibitor protein having substantial sequence identity to amino acid residues 24-886 of SEQ ID NO:2. Examples of suitable chelators include, without limitation, EDTA, meso-2,3-dimercaptosuccinic acid (DMSA), bathophenanthroline-disulfonic acid (BPS), and penicillamine. The amount of each agent effective to inhibit StcE activity will, of course, depend on a variety of factors, including the age, sex, and weight of the individual, for example. To treat other conditions, EDTA has been administered at a rate of 2-3 grams per person over 3 hours. DMSA, which can chelate copper, manganese, molybdenum, and zinc, as well as lead, mercury, cadmium, and arsenic, has been administered for treating heavy metal toxicity, and may be taken orally. Typical dosage protocols range from 500mg/day every other day for a minimum of 5 weeks to 10-30 mg/kg/day using a three-days-on, 11-days-off cycle for a minimum of 8 cycles. However, it is expected that shorter periods of treatment may be indicated. Penicillamine, another chelator primarily used to treat arthritis, can chelate copper, iron, and zinc. It is expected that dosages in the range of 125-750 mg/day may be effective.

[0035] It is also expected that the adverse effects that derive from the proteolytic activity of StcE inhibitor protein may be counteracted by administering to the patient C1-INH in an effective amount. To treat acute attacks of hereditary angioedema (HAE), as little as 25 U/kg has been given to patients (2). For other diseases, initial doses range vary from about 60 U/kg intravenously (i.v.) for patients with vascular leak syndrome to about 6000 U/kg i.v. for patients with severe thermal injury with septic shock. These initial doses can be followed by repeated administration of C1-INH over several days, if necessary. A regimen including an initial dose of 2000-4000 U/kg i.v., followed by additional C1-INH injections (e.g., 1000 U/kg i.v. daily for four days, and variations thereof) has been used to treat septic shock (3). C1-INH can be obtained commercially from various vendors (e.g., Cortex Biochem), or can prepared from pooled human plasma by a variety of protocols (2) (4).

[0036] As described below in the examples, the *stc*E ORF was amplified by PCR, the PCR product comprising the *stc*E ORF was functionally inserted into an expression vector, the recombinant vector was introduced into a bacterial host, and the bacterial host was cultured under conditions suitable for expression of the StcE protein. The StcE protein was purified and characterized as described below in the examples. The above described method of expressing and isolating StcE is a preferred method of obtaining an inhibitor protein of the present invention. However, as one of ordinary skill in the art will appreciate, the protein may be isolated from any other source, including, for example, from culture medium in which bacteria harboring the pO157 plasmid are grown.

[0037] The ability of an isolated polypeptide to bind C1-INH or cleave C1-INH may be assessed using any suitable method, including those methods described in detail in the examples below.

[0038] In order to evaluate the StcE protein for possible cytotoxic effects, a variety of cell types were treated with StcE protein as described in the examples. Cells treated with StcE showed a high degree of aggregation in the presence of serum, but not in the absence of serum.

[0039] Because StcE-mediated aggregation occurred only in cells also treated with serum, the ability of StcE to bind to a specific serum protein was evaluated by Far western blotting using the StcE protein as the probe. An acidic serum protein of about 105 kDa by SDS PAGE was identified as binding to StcE. The target protein was recovered, subjected to limited digestion by an endopeptidase, and the peptide products analyzed by mass spectrometry. The protein to which the StcE protein binds was identified as C1 esterase inhibitor (C1-INH), which serves as a critical inhibitor in the proteolytic cascade involved in complement activation.

[0040] The plasma protein C1-INH is a protease inhibitor that controls the activation of C1, the first component of the complement cascade. The C1 component is made up of three subcomponents: C1q, C1r, and C1s. In the classical pathway of complement activation, C1 binds to an antigen-antibody complex or certain pathogens (e.g., HIV-1)

which causes the proteolytic autoactivation of C1r, which in turn causes the proteolytic activation of C1s. C1-INH inhibits activation of the classical pathway by binding to C1 and inactivating C1r and C1s. In addition to its role in controlling activation of the classical complement pathway, C1-INH inhibits other serine proteases involved in the intrinsic coagulation pathway and kinin-forming system (reviewed in (3)).

[0041] Treatment of serum or purified C1-INH with purified StcE results in the apparent disappearance of C1-INH, putatively as a result of specific proteolytic cleavage of C1-INH by StcE. The predicted StcE amino acid sequence comprises the sequence HEVGHNYGLGH (SEQ ID NO:3) (residues 434-444 of SEQ ID NO:2), which corresponds to the histidine rich active site of metalloproteases (5). Further evidence that StcE may be a metalloprotease is provided by the observation that proteolysis of C1-INH by StcE is reduced in the presence of EDTA or BPS, which chelate divalent metal ions (e.g., Zn^{2+}) required for metalloprotease activity.

[0042] Deficiencies in C1-INH can lead to a variety of diseases. For example, a hereditary deficiency in C1-INH (hereditary angiodema) is characterized by transient, recurrent attacks of intestinal cramps, vomiting and diarrhea. Hereditary defects in production of a different inhibitor of the complement cascade, Factor H, are associated with a form of hemolytic uremic syndrome (HUS) similar to that described for EHEC-mediated HUS.

[0043] Secretion of the putative protease StcE by enterohemorrhagic strains of *E. coli* EHEC may lead to proteolysis of C1-INH and reduction of C1-INH activity. Loss of C1-INH activity may result in unregulated pro-inflammatory or coagulation response that may be responsible for tissue damage in the intestine and kidney of persons infected with EHEC. It is also possible that the StcE serum-dependent cellular aggregation phenotype plays a role in the pathogenesis of HUS because one of the hallmarks of HUS is thrombocytopenia with an accumulation of a large number of platelets in renal microthrombi. The kidneys of those diagnosed with HUS also contain large amounts of deposited fibrin.

[0044] The proteolytic activity of StcE may be a common mode of pathogenesis among some diarrheagenic strains of *E. coli*. Colony blot analysis and amplification of *E. coli* DNA using oligomers specific to the pO157 version of *stcE* indicate that the *stcE* gene is common to all tested strains of *E. coli* associated with bloody colitis and HUS, but the *stc*E gene is not present in enteroinvasive, enterotoxigenic or uropathogenic strains of *E. coli*. However, some closely related strains of enteropathogenic *E. coli* contain *stc*E, which suggests that StcE may be more widely distributed among diarrheagenic *E. coli* than appreciated initially. Additionally, a search of the GenBank database has identified at least one distant homolog to StcE: a Vibrio *cholerae* protein (designated TagA) of unknown function. We can envision a method to screen for similar virulence factors produced by microbes.

[0045] StcE interacts with and cleaves the human serum protein complement C1 inhibitor in a zinc-dependent manner. Inactivation of serine protease inhibitors by this type of virulence factor may result in an unregulated pro-inflammatory and coagulation response that may be responsible for tissue damage in the intestine and kidney in patients infected with enterohemorrhagic strains of *E. coli*.

[0046] The elucidation of StcE functions may result in new targets for chemo-therapeutic or immune-based prevention or treatment of EHEC diseases. Active or passive immune prophylaxis using StcE as an antigen or anti-StcE antibodies may prevent the serious sequalae associated with infections by enterohemorrhagic *E. coli*. Identification of the active site or binding sites between StcE and C1-INH may facilitate the design of drugs capable of preventing proteolysis of C1-INH and the tissue damage that results as a consequence of the loss of C1-INH activity. Administration of C1-INH to patients suffering from hereditary angiodema is a common practice (6). We envision that administration of C1-INH could be an effective therapy for patients suffering from bloody colitis, HUS or thrombotic thrombocytopenic purpura (TTP).

[0047] The following non-limiting examples are intended to be purely illustrative.

EXAMPLES

[0048] Bacterial strains and plasmids.

[0049] A list of bacterial strains and plasmids is found in Table 1. Strains were constructed and plasmids were maintained in either *E. coli* K-12 DH1 or C600 unless otherwise noted. Recombinant DNA manipulations were performed by standard methods.

[0050] Enterohemorrhagic *Escherichia coli* strains EDL933 and EDL933cu (lacking plasmid pO157) and WAM2371 (enteropathogenic *E. coli* strain E2348/69) were provided by Dr. Alison O'Brien of the Uniformed Services University. WAM2035 (C600/pO157) was provided by Dr. Hank Lockman of the Uniformed Services University. WAM2516 (*Citrobacter rodentium* strain DBS100) was provided by Dr. David Schauer of the Massachusetts Institute of Technology. The Diarrheagenic *E.coli* (DEC) collection was a gift from Dr. Tom Whittam of the University of Pennsylvania. WAM2547 was created by transforming pLOF/Km (a gift from Dr. Victor De Lorenzo of the GBF-National Research Centre for Biotechnology, Germany) into the donor strain S17(λpir).

Table 1. Bacterial strains and plasmids used in this study.

Strain	Relevant phenotype or plasmid genotype		Source
C600	*E. coli* K-12		this laboratory
DH1	laboratory strain of *E. coli*		this laboratory
S17(λpir)	*E. coli* donor strain for conjugation		this laboratory
BL21(DE3)	*E. coli* strain for protein overexpression		Novagen
EDL933	wild-type EHEC strain		A. O'Brien
EDL933cu	EHEC strain EDL933 cured of pO157		A. O'Brien
WAM2371	EPEC strain E2348/69		A. O'Brien
WAM2516	*C. rodentium* strain DBS100		D. Schauer
DEC strains	Diarrheagenic *E. coli* collection		T. Whittam
WAM2035	C600/pO157::Tn801	(amp^r)	H. Lockman
WAM2515	C600/pO157::Tn801	(amp^r nal^r)	this study
WAM2297	DH1/pBluescript II SK+	(amp^r)	this laboratory
WAM2547	S17(λpir)/pLOF/Km	(amp^r kan^r)	this study
WAM2553	C600/pWL104	(amp^r kan^r)	this study
WAM2562	DH1/pWL105	(amp^r)	this study
WAM2572	BL21(DE3)/pWL107	(kan^r)	this study
WAM2726	BL21(DE3)/pTEG1	(kan^r)	this study
pLOF/Km	pGP704 carrying miniTn10kan		V. De Lorenzo
pO157	92 kb plasmid of EDL933; Tn801 at base 5413		H. Lockman
pBluescript II SK+	cloning vector		Stratagene
pET24d(+)	6xHis overexpression vector		Novagen
pWL104	pO157::miniTn10kan inserted at base 23772		this study
pWL105	pBluescript II SK+/bases 1-2798 of L7031		this study
pWL107	pET24d(+)/bases 138-2795 of L7031		this study
pTEG1	pWL107 with amino acid change E435D		this study

[0051] WAM2515 is a spontaneous nalidixic acid-resistant mutant of WAM2035. WAM2553 was created as described below, containing a mini-Tn10kan insertion at base 23772 of pO157 (accession #AF074613). This plasmid is designated pWL104. WAM2297 is pBluescript II SK+ in DH1. pWL105 was constructed by amplifying bases 1 to 2798 of the promoter and gene L7031/*stcE* from pO157 by polymerase chain reaction (PCR) using primer pairs 5'-CCCTCGAGTTTACGAAACAGGTGTAAAT-3' (SEQ ID NO:4) and 5'-CCTCTAGATTATTTATATACAACCCTCATT-3' (SEQ ID NO: 5); and cloning the product into the XbaI-XhoI sites of pBluescript II SK+ (Stratagene); WAM2562 is DH1 containing pWL105. pWL107 was constructed by PCR amplification of bases 138 to 2798 of the promoter and gene L7031/*stcE* from pO157 by PCR using primer pairs 5'-CCGAGCTCCGATGAAATTAAAGTAT-CTGTC-3' (SEQ ID NO:6) and 5'-CCTCGAGTTTATATACAACCCTCATTG-3'

(SEQ ID NO:7); and cloning the PCR product into the SacI-XhoI sites of pET-24d(+) (Novagen); WAM2572 is BL21(DE3) (Novagen) transformed with pWL107. The creation of WAM2726 is described below. All chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise.

Cell lines

[0052] All cell lines were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (HyClone) and 10 μg/ml gentamicin at 37°C with 5% CO_2. The human T cell line Jurkat clone E6-1, the human promyelocytic leukemia line HL-60, and the human B cell lymphoma line Raji were obtained from ATCC, the human promyelocytic leukemia line U937 was a gift from Dr. Jon Woods of the University of Wisconsin-Madison, and the human T cell lymphoma line MOLT-4 was a gift from Dr. David Pauza of the University of Wisconsin-Madison.

Aggregation assays.

[0053] Bacterial strains were grown overnight in Lennox L broth (with antibiotic selection when appropriate) at 37°C with agitation. Cultures were washed once with phosphate buffered saline (PBS) and resuspended in 1/10 the original culture volume in PBS. Cultures were lysed in a French Press at 20,000 lbs/in^2. The resulting lysates were spun at 1000 x g to remove debris and protein concentrations were determined by the Bradford protein assay (Bio-Rad). Tissue culture cells were suspended at 10^6 cells/ml in RPMI 1640 and 50 μg/ml gentamicin with 10% FBS or human serum. Fifty μg/ml of lysates or 50-200 ng/ml purified StcE-His (see below) were added to cells and incubated for two hours at 37°C in 5% CO_2. Cells were agitated for one minute to disrupt spontaneous aggregates before visualization. Similar assays were performed in the absence of serum or with ammonium sulfate-precipitated fractions of human serum (see below); cells were washed once in RPMI 1640 and resuspended at 1 x 10^6 cells/ml in RPMI 1640 with 50 μg/ml gentamicin (and human serum fractions, if indicated) before the addition of lysates or StcE-His. When indicated, ethylenediaminetetraacetic acid (EDTA) or bathophenanthroline-disulfonic acid (BPS) were added to the assays at a final concentration of 5 mM.

Identification of *stc*E

[0054] WAM2515 was mated with WAM2547 as described (7). Transconjugants were plated onto LB plates containing 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 50 μg/ml nalidixic acid. Transconjugants were resuspended in 1X TES, washed once with 1X TES, and pO157/pO157::mini-Tn10kan were isolated by midi-prep (Qiagen). pO157/pO157::mini-Tn10kan were transformed into C600 and plated onto LB plates containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. Transformants were grown overnight in Lennox L broth containing 100 μg/ml ampicillin and 50 μg/ml kanamycin at 37°C with agitation and lysates were screened for the ability to aggregate Jurkat cells as described above. pO157::mini-Tn10kan was isolated from clones lacking the ability to aggregate Jurkat cells and the location of the transposable element was identified by sequence analysis. One clone unable to aggregate Jurkat cells was designated WAM2553.

Purification of recombinant StcE-His

[0055] StcE-His was purified according to the manufacturer's instructions (Novagen). Briefly, WAM2572 was induced to produce StcE-His by the addition of IPTG to 1 mM at an O.D. of 0.5 followed by vigorous aeration at 37°C for approximately three hours. The cells were lysed in a French Press at 20,000 lbs/in^2 and the resulting lysate was centrifuged at 20,000 x g for 15 minutes. The insoluble pellet was resuspended in a buffer containing 5 mM imidazole and 6 M urea and the inclusion bodies were solubilized for one hour on ice. This fraction was incubated with nickel-agarose beads (Qiagen) overnight at 4°C, and the beads were washed three times with a buffer containing 60 mM imidazole and 6 M urea. Purified StcE-His was eluted from the beads with a buffer containing 300 mM imidazole and 6 M urea. Eluted StcE-His was dialyzed against three changes of PBS/20% glycerol at 4°C to remove the imidazole and urea. Protein concentration was determined by SDS-PAGE using purified β-galactosidase as a standard. At our request, polyclonal antibodies to purified StcE-His were prepared in rabbits by Cocalico Biologicals, Inc. Briefly, purified StcE-His was electrophoresed on an 8% polyacrylamide gel and stained with Coomassie Brilliant Blue. StcE-His was excised from the gel and injected into rabbits. Rabbits were boosted with StcE-His once a month for six months prior to exsanguinations.

Two-dimensional gel electrophoresis

[0056] Human serum was fractionated by ammonium sulfate precipitation, dialyzed against three changes of RPMI 1640 (Gibco) overnight at 4°C, and protein concentration was determined by Bradford assay (Bio-Rad). When indicated, protein A-sepharose was used to remove fractions of IgG. Two-dimensional electrophoresis was performed according to the method of O'Farrell (8) by Kendrick Labs, Inc. (Madison, WI) as follows: isoelectric focusing was carried out on 25 μg of 30-60% ammonium sulfate-fractionated human serum removed of IgG in glass tubes of inner diameter 2.0 mm using 2.0% pH 3.5-10 ampholines (Amersham Pharmacia Biotech) for 9600 volt-hrs. Fifty ng of an IEF internal standard, tropomyosin, was added to each sample. This protein migrates as a doublet with lower polypeptide spot of MW 33,000 and pI 5.2; an arrow on the stained gel marks its position. The enclosed tube gel pH gradient plot for this set of ampholines was determined with a surface pH electrode.

[0057] After equilibration for 10 min in buffer "0" (10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 0.0625 M Tris, pH 6.8) each tube gel was sealed to the top of a stacking gel that is loaded on the top of a 8% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 hrs at 12.5 mA/gel. The following proteins (Sigma) were added as molecular weight standards to a well in the agarose which sealed the tube gel to the slab gel: myosin (220 kDa), phosphorylase A (94 kDa), catalase (60 kDa), actin (43 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa). These standards appear as bands on the basic edge of the special silver-stained (O'Connell and Stults 1997) 8% acrylamide slab gel. The gel was dried between sheets of cellophane with the acidic edge to the left.

[0058] A similar gel was run as described above with the following differences: 250 μg of 30-60% ammonium sulfate-fractionated human serum was loaded onto the IEF gel; the second dimension was run on a 10% acrylamide slab gel and stained with Coomassie Brilliant Blue.

Far Western blot analysis

[0059] One hundred μg of 30-60% ammonium sulfate-fractionated human serum was run on a two-dimensional gel as described above but without staining. After slab gel electrophoresis the gel for blotting was transferred to transfer buffer (12.5 mM Tris, pH 8.8, 86 mM glycine, 10% methanol) and transblotted to PVDF membrane overnight at 200 mA and approximately 50 volts/gel. The PVDF membrane was blocked with 2% milk (Difco) in buffer AD (20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 0.01% Tween-20) at 4°C. Two μg/ml purified StcE-His was added to the PVDF membrane and allowed to incubate two hours at 4°C. The membrane was washed with buffer AD and blocked with 2% milk in buffer AD. The membrane was reacted with polyclonal anti-His antibody conjugated with horse-radish peroxidase (Santa Cruz), washed with buffer AD, and developed with the LumiGlo chemiluminescence detection system (Kirkegaard & Perry Laboratories). The PVDF membrane was then stripped (62 mM Tris, pH 6.8, 2% SDS, 10 mM β-mercaptoethanol (β-ME), 30 min, 50°C), washed with buffer AD, reacted as above with only the His-HRP antibody, and developed.

Mass spectrometry

[0060] Of the three spots in human serum that reacted with purified StcE-His as identified by Far Western blotting, only the leftmost spot (the most acidic) of approximately 100 kDa was accessible for analysis by mass spectrometry. This spot was cut from the Coomassie Brilliant Blue-stained 10% slab gel and sent to the Protein Chemistry Core Facility at the Howard Hughes Medical Institute/Columbia University for analysis. The spot was digested with endoproteinase Lys-C and analyzed by MALDI-MS. The peptide pattern was compared against known human proteins in the SWISS-PROT database and was identified as plasma protease C1 inhibitor.

Electrophoresis and immunoblot analyses

[0061] Fifty μg whole and ammonium sulfate-precipitated human serum fractions were incubated with 500 ng purified StcE-His in 500 μl buffer AD for two hours at room temperature and precipitated with 10% trichloroacetic acid (TCA) on ice for one hour. Precipitates were collected by centrifugation, resuspended in 1X sample buffer (2% SDS, 10% glycerol, 5% β-ME, 1 mM bromophenol blue, 62 mM Tris, pH 6.8), and

heated to 95-100°C for 5 min prior to electrophoresis on 8% polyacrylamide gels. Separated proteins were transferred to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech) as described (9) for immunoblot analysis. Blots were blocked with 5% milk in TBST (154 mM NaCl, 20 mM Tris, pH 7.6, 0.1% Tween-20), probed with a polyclonal anti-C1 inhibitor antibody (Serotec) and then with HRP-conjugated anti-rabbit secondary antibody (Bio-Rad) before developing as described above.

[0062] Sixteen μg purified C1 inhibitor (Cortex Biochem) were incubated with 4.8 μg purified StcE-His in 480 μl buffer AD at room temperature; 30 μl of the reaction were removed at various time points, suspended in 1X sample buffer, and heated to 95-100°C for 5 min prior to electrophoresis on 8% polyacrylamide gels. Separated proteins were transferred to nitrocellulose and reacted with anti-C1 inhibitor antibody as described above.

[0063] EDL933, EDL933cu, WAM2035, and WAM2553 were grown in Lennox L broth at 37°C overnight, centrifuged, and the culture supernatant was removed. The supernatant was precipitated with ammonium sulfate and the 0-60% fraction was resuspended at 1/100 the original culture volume and dialyzed against three changes of PBS overnight at 4°C. Twenty μl of the dialyzed supernatants and 30 μg of EDL933, EDL933cu, WAM2035, and WAM2553 lysates were suspended in 1X sample buffer and heated to 95-100°C for 5 min prior to electrophoresis on 8% polyacrylamide gels. Separated proteins were transferred to Hybond ECL nitrocellulose and reacted with polyclonal anti-StcE-His antibody, followed by anti-rabbit-HRP secondary antibody.

Casein proteolysis assay

[0064] Various concentrations of StcE-His were incubated with BODIPY FL-conjugated casein for various times using the EnzChek Protease Assay Kit (Molecular Probes, Inc.) and the increase in fluorescence was measured with a fluorimeter as per the manufacturer's instructions.

Lysates *of E. coli* strains carrying pO157 induce the aggregation of transformed human T cell lines in a serum-dependent manner.

[0065] To determine the consequence of pO157- containing *E. coli* products on Jurkat

cells, a human T cell lymphoma line, 50 μg/ml of lysates of strains EDL933, EDL933cu, WAM2035, WAM2371, WAM2516, and C600 were applied to 1 x 10^6 Jurkat cells/ml in RPMI 1640 with 10% FBS and 50 μg/ml gentamicin for two hours at 37°C in 5% CO_2. After agitation for one minute to disrupt spontaneous aggregates, Jurkats were observed for the induction of aggregation. Lysates of *E. coli* strains carrying pO157 induced the aggregation of Jurkat cells while lysates of strains lacking pO157 did not (Figure 1). Lysates of other pathogenic bacteria such as enteropathogenic *E. coli* strain E2348/69 (WAM2371) and *C. rodentium* (WAM2516) capable of inducing the attaching and effacing (A/E) phenotype on intestinal epithelial cells and carrying large virulence plasmids different from pO157 were unable to induce the aggregation of Jurkat cells. To determine whether this effect was specific for Jurkat cells or could induce the aggregation of a broader host cell range, 1 x 10^6 cells/ml in RPMI 1640 with 10% FBS and 50 μg/ml gentamicin of another human T cell lymphoma line, MOLT-4, two human promyelocytic leukemia cell lines, HL-60 and U937, and a human B cell lymphoma line, Raji, were treated with 50 μg/ml of EDL933 and WAM2035 lysates for two hours at 37°C in 5% CO_2. pO157-containing lysates aggregated MOLT-4 cells but not HL-60, U937, or Raji cells (data not shown), indicating T cell specificity for the phenotype.

[0066] To determine the serum requirement for the induction of aggregation, 50 μg/ml of lysates of EDL933 and WAM2035 were applied to 1 x 10^6 Jurkat cells/ml with 10% human serum and 50 μg/ml gentamicin for two hours at 37°C in 5% CO_2. As seen with FBS, pO157-containing lysates were able to induce the aggregation of Jurkat cells in the presence of human serum. However, EDL933 and WAM2035 lysates were unable to induce the aggregation of Jurkat cells under the same conditions in the absence of serum. To further characterize the component(s) of human serum responsible for mediating Jurkat cell aggregation in the presence of StcE, we fractionated human serum by ammonium sulfate precipitation followed by dialysis in RPMI 1640. We found that 0-30% and 30-60%, but not 60-100%, ammonium sulfate-precipitated human serum was able to mediate aggregation of Jurkat cells in the presence of StcE. This indicates a factor or factors in serum is required for the aggregation of Jurkat cells when treated with lysates of pO157-containing bacteria.

Identification and cloning of *stcE*

[0067] To localize the gene(s) on pO157 responsible for the induction of aggregation of human T cell lines, we subjected pO157 to mutagenesis using a minitransposon. Lysates of recombinant strains of *E. coli* containing pO157 mutagenized with mini-Tn10kan were tested for the ability to aggregate Jurkat cells in RPMI 1640 with 10% FBS and 50 μg/ml gentamicin. pO157::mini-Tnl0kan was isolated from clones whose lysates were unable to induce the aggregation of Jurkat cells. The location of the transposon insertion in WAM2553 was determined by sequence analysis and mapped to position 23772 of pO157. The open reading frame in which the transposon inserted was designated L7031 (10) and is located immediately 5' to the general secretory apparatus on pO157. L7031/*stc*E was amplified and cloned into the XbaI-XhoI sites of pBluescript II SK+. Lysates of WAM2562 induced aggregation of Jurkat cells in the presence of serum, whereas lysates of WAM2297 (DH1 carrying pBluescript II SK+) did not, which confirms that the *stc*E gene is responsible for the phenotype.

[0068] Based on sequence analysis, we concluded that the translational start site for StcE was more likely to begin at base 138 than at base 102 (10). We therefore amplified the coding sequence for *stc*E from bases 138 to 2798 by PCR and cloned the gene in frame with a 6xHis-tag at the 3' end of the fusion in pET24d(+). We were able to overexpress and purify a recombinant his-tagged form of StcE (StcE-His) (Figure 2); this purified fusion protein was able to aggregate Jurkat cells in the presence of serum at a variety of concentrations (data not shown).

Localization and characterization of StcE

[0069] Using antiserum to StcE-His, we performed immunoblot analysis to examine the expression and secretion of StcE by *E. coli.* StcE is expressed by *E. coli* strains carrying pO157 at 37°C in Lennox L broth but not in strains lacking pO157 or harboring a transposon insertion in *stcE* (Figure 3). Additionally, StcE is released into the culture supernatant by strains carrying pO157 under the same growth conditions (Figure 3). As StcE contains a putative cleavable N-terminal signal sequence, it is possible that StcE is

actively released from the bacterium by the general secretory apparatus encoded on pO157.

[0070] StcE-mediated Jurkat cell aggregation is inhibited by the addition of ion chelators such as EDTA, a broad chelator of divalent cations, and BPS, a chelator specific for zinc and iron ions (data not shown). This suggests that StcE has a requirement for one or more divalent cations, most likely zinc. This is supported by the presence of an exact match to the histidine-rich consensus active site for metalloproteases, which coordinate zinc ions for activity (see discussion).

[0071] StcE-His interacts with a human serum protein(s) of approximately 105 kDa. To identify the factor(s) in human serum responsible for mediating Jurkat cell aggregation in the presence of StcE, the 30-60% ammonium sulfate-precipitated fraction of human serum was separated on a two-dimensional gel and transferred to a PVDF membrane. Using purified StcE-His as a probe, we performed Far Western blot analysis on the PVDF membrane, detecting any interactions between StcE-His and human serum proteins with an HRP-conjugated anti-His antibody. We found that StcE-His interacts with three spots of approximately 105 kDa ranging from very acidic to very basic in isoelectric point (data not shown). Probing the same membrane with only the HRP-conjugated anti-His antibody revealed that the three spots of approximately 105 kDa were specific for StcE-His (data not shown).

[0072] To identify these proteins, the 30-60% ammonium sulfate-precipitated fraction of human serum was removed of IgG and separated on another two-dimensional gel and either special silver stained or stained by Coomassie Brilliant Blue. The most acidic of the three spots (the leftmost spot) was well isolated from other proteins and excised from the Coomassie Brilliant Blue-stained gel. This spot was digested by endoproteinase Lys-C and analyzed by MALDI-MS. A comparison of the resulting peptide pattern with known human proteins in the SWISS-PROT database revealed a match with human plasma protease C1 inhibitor.

Cleavage of C1 inhibitor by StcE-His

[0073] To confirm the interaction between StcE and human C1 inhibitor and to test the possibility that StcE may proteolyze C1 inhibitor, whole and ammonium sulfate-precipitated fractions of human serum were mixed with StcE-His, separated by SDS-PAGE, and transferred to nitrocellulose for immunoblot analysis. Using an anti-human C1 inhibitor antibody, we detected the presence of C1 inhibitor in samples lacking StcE-His and the absence of C1 inhibitor in samples containing StcE-His (Figure 4). As predicted by Jurkat cell aggregation, the 0-30% and 30-60% ammonium sulfate-precipitated fractions of human serum were enriched for C1 inhibitor compared to the 60-100% fraction. After treatment with StcE-His, however, little to no C1 inhibitor could be detected in any of the fractions. The addition of EDTA or BPS to the mixture prevented the disappearance of C1 inhibitor from the serum samples, indicating a specific requirement for divalent cations, most likely zinc, for StcE activity (data not shown).

[0074] To confirm that the proteolysis of C1 inhibitor was a direct result of an interaction with StcE-His, we mixed purified human C1 inhibitor with StcE-His and removed aliquots of the reaction at various time points for analysis by immunoblot. Using an anti-human C1 inhibitor antibody, we detected the disappearance of a 105 kDa band corresponding to full-length C1 inhibitor and the appearance of an approximately 60 kDa cleavage product in a time-dependent manner (Figure 5).

Examination of patient fecal filtrates for StcE

[0075] Freshly passed stool samples from children with culture-positive *E. coli* O157:H7 (n=6), *Campylobacter jejuni* (n=2), *Shigella* B (n=2), or *Clostridium difficile* (n=2) infections were diluted 1:10 in PBS and passed through a 0.45 μm filter. Thirty μl of thawed filtrate was suspended in 1X sample buffer, heated (95-100°C for 5 min) and electrophoresed on 8% polyacrylamide gels. Separated proteins were transferred to nitrocellulose and probed with a polyclonal antibody to StcE-His as described above. Twenty μl of the same samples was added to 1 X 10^6/ml Jurkat cells in 10% FCS and

gentamicin (50 μg/ml) for 24 hours at 37°C in 5% CO_2 to determine the ability of the filtrates to aggregate Jurkat cells.

Construction and analyses of StcE E435D-His mutant

[0076] The StcE E435D-His mutant was created using the PCR-based method of overlap extension (Horton *et al.* 1993). The first two PCR reactions were (i) *stcE* top strand primer 587 (5'-CCGCTCCGGTGAACTGGAGAATA-3') (SEQ ID NO:8) with its partner mutagenic primer 592 (5'-GACCATAATTATGACCAACATCATGAC-TGA-3') (SEQ ID NO:9) and (ii) *stcE* bottom strand primer 573 (5'-CCTTATCTGCG-GAGGCTGTAGGG-3') (SEQ ID NO:10) with its partner mutagenic primer 574 (5'-TGAGTTCAGTCATGATGTTGGTCATAATTAT-3') (SEQ ID NO:11). Each reaction used 50 pM of each primer, about 100 ng of template DNA, and Deep Vent polymerase (New England Biolabs) in a 100 μl reaction. The reactions were run in a thermocycler under appropriate conditions (11) and the resulting products were purified on a 1% agarose gel using the QIA-quick Gel Extraction Kit (Qiagen). The next PCR reaction contained 5 μl each of the gel-purifed fragments, along with the *stcE* primers 587 and 573 and Deep Vent polymerase in a 100 μl reaction. The PCR products were gel-purified as above and cut with the restriction endonucleases *Pme*I and *BsrG*1. pWL107 was also cut with *Pme*I and *BsrG*1 and the mutant PCR product was ligated into pWL107, creating pTEG1. The base substitution was confirmed by sequence analysis. pTEG1 was transformed into *E. coli* strain BL21(DE3) to create WAM2726 and StcE E435D-His was overexpressed and purified from this strain as described above. The purified protein was then analyzed for its ability to aggregate Jurkat cells as described above.

[0077] Purified C1-INH (one μg) was mixed with or without StcE-His (one μg) or StcE E435-His (one μg) overnight at room temperature in 500 μl buffer AD, precipitated with TCA (to 10%), electrophoresed on an 8% polyacrylamide gel, and transferred to nitrocellulose before analysis by immunoblot with an anti-C1-INH antibody as described above.

[0078] Purified C1-INH (500 ng) and human serum (50 μg) were electrophoresed on an 8% polyacrylamide gel in duplicate and the separated proteins were transferred to nitrocellulose for Far Western analysis. Essentially the same protocol was followed as described above with the following difference: one blot was probed with purified StcE-His (2 μg/ml) and the other with purified StcE E435D-His (2 μg/ml).

Colony blot analysis

[0079] A one kb fragment of *stcE* was PCR amplified from pO157 using the primers *stcE*5'846 (5'-GAGAATAATCGAATCACTTATGCTC-3') (SEQ ID NO:12) and *stcE*3'1773 (5'-CGGTGGAGGAACGGCTATCGA-3') (SEQ ID NO:13) under standard reaction conditions. The PCR product was purified on a 1% agarose gel using the QIA-quick Gel Extraction Kit (Qiagen) and fluorescein-labeled using the ECL random prime labeling system (Amersham Life Science). Bacterial strains from the DEC collection, EDL933, and EDL933cu were patched onto sterile Magna Lift nylon transfer membranes (Osmonics) on LB plates and grown overnight at room temperature. Colonies were lysed by placing the membranes on 3MM Whatman paper soaked in 0.5 M NaOH. Neutralization was performed by placing the membranes first on 3MM Whatman paper soaked in 1 M Tris, pH 7.5 and then on 3MM Whatman paper soaked in 0.5 M Tris, pH 7.5/1.25 M NaCl. DNA was then crosslinked using a UV stratalinker. The blots were pre-hybridized in Church buffer (0.5 M dibasic sodium phosphate, pH 7.3, 7% SDS, 1% BSA, 1 mM EDTA) at 65°C for one hour before the addition of the labeled probe. Hybridization proceeded overnight at 65°C. The membranes were then washed at 65°C in 1 X SSC/0.1% SDS for 15 minutes and then in 0.5X SSC/0.1% SDS for 15 minutes. The membranes were incubated with an anti-fluorescein labeled, HRP-conjugated antibody. The membrane was developed using the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard and Perry Laboratories).

PCR analysis of *stcE*

[0080] Oligonucleotides were designed to amplify by PCR regions of *stcE* to cover the length of the ~2.8 kbp promoter and gene. Primers *stcE*5'1 (5'-TTTACGAAACA-GGTGTAAATATGTTATAAA-3') (SEQ ID NO:14) and *stcE*3'845 (5'-CAGTTCACCG-GAGCGGAACCA-3') (SEQ ID NO:15) covered the first third,

*stcE*5'846 and *stcE*3'1773 covered the middle third, and *stcE*5'1774 (5'-GCTTCAGC-AAGTGGAATGCAGATAC-3') (SEQ ID NO:16) and *stcE*3'2798 (5'TTATTTAT-ATACAACCCTCATTGACCTAGG-3') (SEQ ID NO:17) covered the final third. Genomic DNA was isolated from *E. coli* strains DEC3A-E, DEC4A-E, DEC5A-E, EDL933, and EDL933cu using the Wizard Genomic DNA Purification Kit (Promega) as per the manufacturer's instructions. PCR was performed using 20 pM of each primer, about 100 ng of template DNA, and Deep Vent polymerase (New England Biolabs) in a 100 μl reaction. The reactions were run in a thermocycler under standard conditions. PCR products were electrophoresed on 1% agarose gels and visualized with ethidium bromide.

Isolation and analyses of bacterial culture supernatants

[0081] *E. coli* strains DEC3A-E, DEC4A-E, DEC5A-E, EDL933, and EDL933cu were grown in Lennox L broth at 37°C overnight. Culture supernatants were harvested by centrifugation at 4°C for 15 minutes at 10,000 x g and filtered through a 0.45μm filter. Supernatants were precipitated with ammonium sulfate to 75% saturation. The precipitates were centrifuged for 15 minutes at 16,000 x g at 4°C and resuspended in buffer AD. The resuspended precipitates were dialyzed against three changes of AD buffer overnight to remove residual ammonium sulfate.

[0082] Purified CI-INH (one μg) was mixed with 200μl of ammonium sulfate-precipitated culture supernatants at room temperature overnight in a total volume of 500 μl buffer AD before precipitation with TCA (to 10%) and electrophoresis on 8% polyacrylamide gels. Separated proteins were transferred to nitrocellulose and immunoblot analysis was performed with an anti-C1-INH antibody as described above. Culture supernatants alone were separated on 8% polyacrylamide gels and transferred to nitrocellulose before immunoblot analysis was performed using an anti-StcE-His antibody as described above.

Specificity of StcE-His for C1-INH

[0083] To evaluate the specificity of StcE-His, potential target proteins (listed in Table 2), target protein (2 μg) was mixed with either StcE-His (1.28 μg) or *Pseudomonas aeruginosa* elastase (20 ng) (Calbiochem EC# 3.4.24.26) overnight at 37°C in 500 μl

buffer AD (20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 0.01% Tween-20) and precipitated with TCA (to 10%) prior to electrophoresis on 8-10% polyacrylamide gels. Proteins in the gels were then Coomassie-stained or transferred to nitrocellulose for immunoblot analysis as above.

[0084] StcE is able to cleave both purified and serum-associated C1-INH. Only C1-INH was cleaved by StcE-His; the sizes and staining intensities of all other potential substrates were the same in the presence and absence of StcE-His. In contrast, elastase degraded most of the proteins tested. Elastase treatment of C1-INH normally produces an inactive 95 kDa product (12), whereas treatment of C1-INH with StcE-His results in ~60-65 kDa C1-INH fragment(s) (Figure 6). This indicates that the StcE cleavage site of C1-INH is distinct from that used by elastase. We employed a sensitive fluorimetric assay based on the digestion of a BODIPY FL-labeled casein substrate (EnzChek, Molecular Probes) to analyze further the ability of StcE-His to act as a non-specific endoprotease. Serial two-fold dilutions of StcE-His or *P. aeruginosa* elastase were mixed with the casein substrate per the manufacturer's instructions before fluorescent measurement of casein degradation. StcE-His was unable to degrade casein even at high protein concentrations (up to 6.4 μg/unit of volume), while elastase was able to act on casein at lower concentrations (range: 0.5 ng to 1 μg/unit of volume) (data not shown).

Table 2. Proteolysis of substrates incubated with StcE-His or *P. aeruginosa* elastase

Substrate	StcE	Elastase
C1 inhibitor (Cortex Biochem, San Leandro, CA)	+	+
α2-antiplasmin (Calbiochem, San Diego, CA)	-	+
α1-antitrypsin (Sigma, St. Louis, MO)	-	+
α1-antichymotrypsin (Sigma, St. Louis, MO)	-	+
antithrombin (Enzyme Research Labs, South Bend, IN)	-	+
α2-macroglobulin (Calbiochem, San Diego, CA)	-	+
von Willebrand factor (gift from Dr. D. Mosher, UW-Madison)	-	N.D.
collagen IV (Rockland, Gilbertsville, PA)	-	-
fibronectin (Calbiochem, San Diego, CA)	-	+
serum albumin (New England Biolabs, Beverly, MA)	-	N.D.
IgA1 (Cortex Biochem, San Leandro, CA)	-	+
Elastin (Sigma, St. Louis, MO)	-	+
Gelatin (BioRad, Hercules, CA)	-	+

N.D. = not done

Two μg of the indicated protein substrates were mixed with 1.28 μg StcE-His or 20 ng *P. aeruginosa* elastase overnight at 37°C prior to electrophoresis by SDS-PAGE and staining with Coomassie Brilliant Blue. StcE was unable to digest any of the proteins tested other than C1-INH, while *P. aeruginosa* elastase had activity against a broad range of targets.

Detection of StcE in feces.

[0085] The Shiga-like toxin has been identified in the feces of patients infected with *E. coli* O157:H7 (13, 14). To demonstrate that StcE is produced *in vivo* during an *E. coli* O157:H7 infection, we examined fecal filtrates collected from patients with *E. coli* O157:H7 and non-*E. coli* O157:H7-mediated diarrhea for the presence of StcE antigen and activity. Twelve fecal samples were diluted in PBS and filtered before analysis by immunoblot with polyclonal antibodies to StcE-His. A strongly reactive band with a molecular weight similar to StcE was present in the filtrate from one child infected with *E. coli* O157:H7 (Figure 7, sample 2). Because StcE is able to mediate the aggregation of T cells, we examined the ability of the twelve fecal filtrates to aggregate Jurkat cells. Twenty μl of each filtrate was added to 5 x 10^5 Jurkat cells in the presence of 10% FCS. The one sample that contained a StcE-reactive species aggregated Jurkat cells to the same extent as 50 ng/ml purified StcE-His; all other samples were negative in this assay, even after 24 hours of incubation (data not shown).

StcE contains a zinc metalloprotease active site.

[0086] As the predicted StcE amino acid sequence has a consensus Zn^{2+}-ligand binding site of metalloproteases (434: HEVGHNYGLGH) (SEQ ID NO:3), we examined the possibility that the glutamic acid residue at position 435 is critical for the proteolysis of C1-INH. This amino acid in other zinc metalloproteases acts as the catalytic residue for proteolysis (15) (16), and other researchers have shown that a conservative amino acid substitution from glutamic to aspartic acid disrupts the activity of the protease while maintaining its structure (15). By introducing a single change in the sequence of *stcE* at base 1442 from an A to a T, we created the same mutation and examined the ability of the recombinant mutant (StcE E435D-His) to digest C1-INH. While StcE-His is able to degrade C1-INH, we observed no such cleavage with the mutant protein (Figure 8A) under the same conditions. Indeed, StcE E435D-His was unable even to bind to C1-INH or a similarly sized protein in human serum as determined by Far Western analysis (Figure 8B), suggesting that glutamic acid 435 is necessary for both binding and cleavage of C1-INH. The StcE-mediated aggregation of Jurkat cells was also affected by the E435D mutation. Jurkat cells will aggregate in response to StcE-His at concentrations as low as 1 ng/ml, while cells treated with as much as 200 ng/ml StcE E435D-His did not aggregate (data not shown). Thus, the glutamic acid residue at position 435 is critical for StcE-mediated aggregation of Jurkat cells, as well as proteolysis of and binding to C1-INH.

Detection of *stcE* among diarrheagenic *E. coli* strains.

[0087] In order to establish the prevalence of *stcE* among other pathogenic strains of *E. coli*, we examined the Diarrheagenic *E. coli* (DEC) collection, a reference set of 78 *E. coli* strains provided by Dr. Tom Whittam of the University of Pennsylvania, for the presence of *stcE*. This collection contains a variety of enterohemorrhagic, enteropathogenic, and enterotoxigenic *E. coli* strains of different serotypes isolated from humans, non-human primates, and other mammals that are associated with disease symptoms, including diarrhea, hemorrhagic colitis, or HUS. The DEC collection is divided into 15 subgroups based on electrophoretic type, which is indicative of the genetic similarity of one strain to another. By using colony blot analysis, we found that all O157:H7 strains of *E. coli* (DEC3 and DEC4) contain DNA that hybridizes with an

internal one kb region of *stcE* (Table 3). Surprisingly, three of five enteropathogenic O55:H7 strains of *E. coli* (DEC5A, C, & E) also hybridized with the *stcE* probe. Because O157:H7 strains are thought to have evolved from an O55:H7 predecessor, this result suggests a source of the *stcE* gene for current O157:H7 strains of *E. coli*. None of the other strains in the DEC collection hybridized with the *stcE* probe by colony blot analysis.

[0088] To confirm the presence of the gene among the *stcE*-positive groups in the DEC collection, we isolated genomic DNA from DEC3A-E, DEC4A-E, DEC5A-E, EDL933, and EDL933cu and used oligonucleotide pairs designed to amplify regions of *stcE* by PCR. Three primer sets were chosen to amplify *stcE* and its promoter from bases 1-845, 846-1773, and 1774-2798. An appropriately-sized PCR product was amplified with all three primer pairs from EDL933, DEC3A-E, and DEC4A-E (Figure 9). Appropriately sized products were obtained with primer pairs 846-1773 and 1774-2798 for DEC5 A, C, and E, but there were no products with primer pair 1-845 from these strains. It is possible that this region of *stcE*, which includes the putative promoter, is sufficiently different from *stcE* found on pO157 to prevent priming and amplification. DEC5B and D were negative for all three reactions.

Table 3. Incidence of *stcE* and its product in the DEC collection

DEC Number	Predominant Serotype	Disease Category	Number of *stcE* positive	Number of StcE positive
1A-E	O55:H6	EPEC	0/5	ND
2A-E	O55:H6	EPEC	0/5	ND
3A	**O157:H7**	**EHEC**	+	+
3B	**O157:H7**	**EHEC**	+	+
3C	**O157:H7**	**EHEC**	+	+
3D	**O157:H7**	**EHEC**	+	+
3E	**O157:H7**	**EHEC**	+	+
4A	**O157:H7**	**EHEC**	+	+
4B	**O157:H7**	**EHEC**	+	+
4C	**O157:H7**	**EHEC**	+	+
4D	**O157:H7**	**EHEC**	+	+
4E	**O157:H7**	**EHEC**	+	+
5A	**O55:H7**	**EPEC**	+	+
5B	O55:H7	EPEC	-	-
5C	*O55:H7*	*EPEC*	+	-
5D	O55:H7	EPEC	-	-
5E	**O55:H7**	**EPEC**	+	+
6A-E	O111:H12	EPEC	0/5	ND
7A-E	O157:H43	ETEC	0/5	0/5
8A-E	O111:H8	EHEC	0/5	ND
9A-E	O26:H11	EHEC	0/5	ND
10A-E	O26:H11	EHEC	0/5	ND
11A-E	O128:H2	EPEC	0/5	ND
12A-E	O111:H2	EPEC	0/5	ND
13A-E	O128:H7	ETEC	0/5	ND
14A-E	O128:H21	EPEC	0/5	ND
15A-E	O111:H21	EPEC	0/5	ND

Using a one kb probe internal to *stcE*, colony blot analyses were performed to determine which strains in the DEC collection contained *stcE*. Strains that were positive for the gene were checked for secretion of StcE as well proteolytic activity against C1-INH. Strains in bold contained the gene and produced the protein. Strains in italics contained the gene but lacked detectable protein. All other strains in the DEC collection were negative for *stcE*. ND = not done.

[0089] Because previous experiments showed that StcE is released into the culture medium by EDL933 (Figure 3), we examined whether the *stcE*-positive strains from the DEC collection also release StcE into the culture medium. We grew DEC3A-E, DEC4A-E, DEC5A-E, EDL933, and EDL933cu overnight in Lennox L broth at 37°C, harvested and concentrated the culture supernatants 100-fold. By immunoblot analysis we were able to detect StcE-reactive antigen in the supernatants of all *stcE*-positive

strains and DEC5C (Figure 10). The intensity of the reactive band varied from strain to strain and seemed to be stronger in the DEC3 group. To test if the bacterial-conditioned culture supernatants contained C1-INH proteolytic activity, we mixed purified C1-INH with the supernatants overnight and examined substrate cleavage by immunoblot. Again, all *stcE*-positive strains except DEC5C were able to degrade C1-INH (Figure 11). Interestingly, DEC5B converted C1-INH from a single band to a doublet; this is unlikely to be related to StcE activity and the significance of this is unknown. It appears that DEC5C is unable to release StcE into the culture medium, although it contain *stcE*-like DNA. This may be due to a lack of expression of the gene or release of the protein from the cell.

1. Perna, N. T., Plunkett III, G., Burland, V., Mau, B., Glasner, J. D., Rose, D. J., Kirkpatrick, H. A., Postal, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E. J., Davis, N. W., Lim, A., Dimalanta, E. T., Potamousis, K. D., Apodaca, J., Anantharaman, T. S., Lin, J., Yen, G., Schwartz, D. C., Welch, R. A. & Blattner, F. R. (2001) *Nature* **409,** 529-533.

2. Waytes, A. T., Rosen, F. S. & Frank, M. M. (1996) *N. Engl. J. Med.* **334,** 1630-1634.

3. Caliezi, C., Wuillemin, W. A., Zeerleder, S., Redondo, M., Eisele, B. & Hack, C. E. (2000) *Pharmacol. Rev.* **52,** 91-112.

4. Poulle, M., Burnouf-Radosevich, M. & Burnouf, T. (1994) *Blood Coagulation & Fibrinolysis* **5,** 543-9.

5. Kuno, K., Terashima, Y. & Matsushima, K. (1999) *Journal of Biological Chemistry* **274,** 18821-6.

6. Gadek, J. E., Hosea, S. W., Gelfand, J. A., Santaella, M., Wickerhauser, M., Triantaphyllopoulos, D. C. & Frank, M. M. (1980) *N. Engl. J. Med.* **302,** 542-546.

7. Lorenzo, V. D. & Timmis, K. N. (1994) in *Bacterial Pathogenesis*, eds. Clark, V. L. & Bavoil, P. M. (Academic Press, San Diego), Vol. 235, pp. 386-405.

8. O'Farrell, P. H. (1975) *J Biol Chem* **250,** 4007-21.

9. Bauer, M. E. & Welch, R. A. (1996) *Infect. Immun.* **64,** 167-175.

10. Burland, V., Shao, Y., Perna, N. T., Plunkett, G., Sofia, H. J. & Blattner, F. R. (1998) *Nucleic Acids Research* **26,** 4196-4204.

11. Roesch, P. L. & Blomfield, I. C. (1998) *Molecular Microbiology* **27,** 751-61.

12. Catanese, J. & Kress, L. F. (1984) *Biochim. Biophys. Acta* **789,** 37-43.

13. Karmali, M. A., Petric, M., Steele, B. T. & Lim, C. (1983) *Lancet* **1,** 619-620.

14. Caprioli, A., Luzzi, I., Gianviti, A., Russmann, H. & Karch, H. (1995) *J. Med. Microbiol.* **43,** 348-353.

15. Jiang, W. & Bond, J. S. (1992) *FEBS Lett* **312,** 110-114.

16. Jung, C.-M., Matsushita, O., Katayama, S., Minami, J., Sakurai, J. & Okabe, A. (1999) *J. Bact.* **181,** 2816-2822.